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Ethnic variation in *Hpa* I endonuclease cleavage patterns of human mitochondrial DNA

(polymorphism/evolution/population genetics/restriction endonuclease)

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ABSTRACT **The mtDNAs of 235 individuals from five ethnic groups were analyzed for restriction site variation by digestion with restriction endonuclease *Hpa* I, Southern transfer, and hybridization with ^{32}P-labeled human mtDNA. Six different cleavage patterns (morphs) were found, all of which could be related to each other by single nucleotide substitutions. Differences were found in the frequency of these morphs among the populations. The largest difference observed was in the frequency of the morph most common in Caucasians and Orientals compared to the frequency of that found in Africans. This difference apparently originated by the sequence change G-T-C-A-A-C to G-T-T-A-A-C. This alteration permitted recognition by *Hpa* I but did not alter the amino acid sequence. Two other observed differences were due to separate substitutions occurring in the ribosomal RNA genes. Comparison with primate data shows that the morph with two fragments, found in 12.5% of Oriental and 4% of Bantu samples, might be the ancestral type common to all hominoids. These two conserved sites were localized in tRNA genes in the anticodon loop. Assuming that the two-fragment morph is ancestral, this finding is consistent with previous data suggesting that Asia is genetically central to the radiations that are thought to have given rise to the human ethnic groups.**

Human mtDNA is a closed-circular molecule of 16.569 kilobases (kb) present in up to 9000 copies per mammalian cell (1). The coding sequence has been found to be virtually saturated by a large and small rRNA, 11 or 12 amino acid coding sequences, and 22 tRNA genes. The tRNAs commonly separate the genes, with few if any unused nucleotides intervening (2–8). Unlike nuclear genes, the human mtDNA has been found to be inherited exclusively from the mother (9, 10).

Restriction endonuclease analysis of multicellular animal mtDNA has become a powerful method in the detection of sequence variation. As such, it has provided an excellent tool for studying evolutionary relationships in animals (2, 11–14) and man (15).

In this study we used mtDNA extracted from blood platelets or peripheral blood to analyze the frequency of restriction site variations in different human ethnic groups. Using *Hpa* I, for which three different morphs had previously been described (15), we have found three additional morphs and observed significant variation in their frequencies. The probable nature of the nucleotide substitutions has been inferred in two cases, and the observed cleavage patterns have been found to be consistent with a simple cladogram.

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MATERIAL AND METHODS

Populations. Blood samples were of the following origins: 54 Caucasians, U.S. Whites and Europeans; 46 Orientals, Taiwan and Mainland Chinese and 2 Japanese; and 133 Africans, 44 Aka Pygmies from Central African Republic, 41 San-Bushmen from Botswana, and 48 Bantu-speaking Africans from Johannesburg, of which 25 were Zulu and 23 were from other local tribes.

Sample Collection. For the frequency analysis, mtDNA from Bushmen and Bantu blood platelets (9) or total DNA from Caucasian and Oriental buffy coats was used. For Pygmies, unwashed "red cell" samples were used. These contained platelets and white cells and were prepared by centrifugation of blood collected in acid/citrate/dextrose (ACD) that had been stored in liquid nitrogen for over 12 years. To test possible tissue differences in mtDNA restriction fragments, DNA was extracted from both platelets and buffy coats of selected Caucasians, Orientals, and Bushmen. All individuals were chosen to be unrelated on the maternal side, using pedigree data.

DNA Isolation. Extraction of mtDNA from platelets has been described (9). When platelets were not available, total DNA was extracted from the buffy coats by the methods of Kan and Dozy (16, 17) with the modification that, after the first phenol extraction, the aqueous phase was dialyzed overnight against 10 mM Tris·HCl (pH 7.4)/1 mM EDTA. This change markedly facilitated restriction endonuclease digestions of whole cell DNA.

For DNA extraction from the "red cell" samples, the buffy coat isolation procedure was used except that approximately 0.15 g of the sample was initially suspended in a final volume of 400 μl of 100 mM NaCl/50 mM Tris·HCl (pH 7.4)/1 mM EDTA. The last dialysis after the final phenol extraction was omitted, and the samples were instead extracted with ether, precipitated with ethanol, dried under reduced pressure, and resuspended in 100 μl of 10 mM Tris·HCl (pH 7.4)/1 mM EDTA.

Tissue of Origin. In previous work with humans, mtDNA was collected from placentas (15), from platelets (9), or from skin fibroblasts (10). However, no direct comparison of mtDNA restriction patterns has been made for the different tissues within an individual. Consequently, mtDNA patterns were compared for selected individuals having each of the different *Hpa* I morphs. In every individual, the pattern was found to be the same in platelets and white cells (data not shown).

Restriction Analysis. One microgram of total DNA, approx-

Abbreviation: kb, kilobase(s).

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imately 0.5 μg of "red cell" DNA, or about 0.2 μg of platelet DNA (9) was used for the restriction endonuclease digestions. The conditions and buffers used were those recommended by the manufacturer except that bovine serum albumin was omitted. All enzymes used were purchased from Bethesda Research Laboratories (Rockville, MD).

After 4 hr of digestion with a 4-fold excess of enzyme, the samples were heated at 65°C for 10 min to inactivate the enzyme. Bromocresol purple was used as a dye marker, glycerol was added to a final concentration of 10% (vol/vol), and the digested fragments were separated on a 0.8% horizontal agarose slab gel at 1.5 V/cm. The fragments were then transferred to a nitrocellulose filter (Millipore) (18) and hybridized with mtDNA purified from HeLa S3 cells (19) nick translated to a specific activity of 2×10^8 cpm/μg (20, 21). Fragments were then visualized by autoradiography. λ phage DNA digested with *Sma* I or double digested with *Hin*dIII and *Eco*RI was used as size standards (22).

RESULTS

Patterns of Cleavage with *Hpa* I. Six different cleavage patterns were detected at various frequencies in the different populations. The number of fragments ranged from two to four. An autoradiogram of all the patterns is shown in Fig. 1, and the characteristics of the morphs obtained are listed in Table 1. Of the fragments that were observed, one of them, corresponding to fragment $\overline{ab}$, 4.21 kb (see Fig. 1), was found in all human mtDNA. The first three morphs correspond to those described by Brown (15).

Large sequence rearrangements in any of the morphs are not likely to explain these patterns, because each morph has been double digested with *Bam*HI and *Hin*dIII. None showed differences in this pattern (data not shown). For all morphs, the sum of all fragments is constant, within the 2% error of these estimates, thus excluding large insertions or deletions as an explanation for these changes.

Table 1. mtHpa I morphs

Morph	Cleavage sites	Fragments	Fragment sizes, kb
mtHpa I-1	a, b	$\overline{ab}$	4.21
		$\overline{ba}$	12.30
mtHpa I-2	a, b, c	$\overline{ab}$	4.21
		$\overline{bc}$	2.51
		$\overline{ca}$	10.03
mtHpa I-3	a, b, c, d	$\overline{ab}$	4.21
		$\overline{bc}$	2.51
		$\overline{cd}$	7.88
		$\overline{da}$	1.90
mtHpa I-4	a, b, c, e	$\overline{ab}$	4.21
		$\overline{be}$	1.78
		$\overline{ec}$	0.51
		$\overline{ca}$	10.03
mtHpa I-5	a, b, c, f	$\overline{ab}$	4.21
		$\overline{bc}$	2.51
		$\overline{cf}$	4.60
		$\overline{fa}$	5.28
mtHpa I-6	a, b, g	$\overline{ab}$	4.21
		$\overline{bg}$	8.65
		$\overline{ga}$	3.54

Probable Molecular Basis of Mutations. Three *Hpa* I sites generating morph mtHpa I-2 have already been mapped (11, 23, 24). These are designated a, b, and c in Fig. 2. They have been used as references to map the remaining sites. Fig. 2 also includes the map for the *Hinc*II sites (11), which helped to locate some of the new *Hpa* I sites. It is known that *Hpa* I

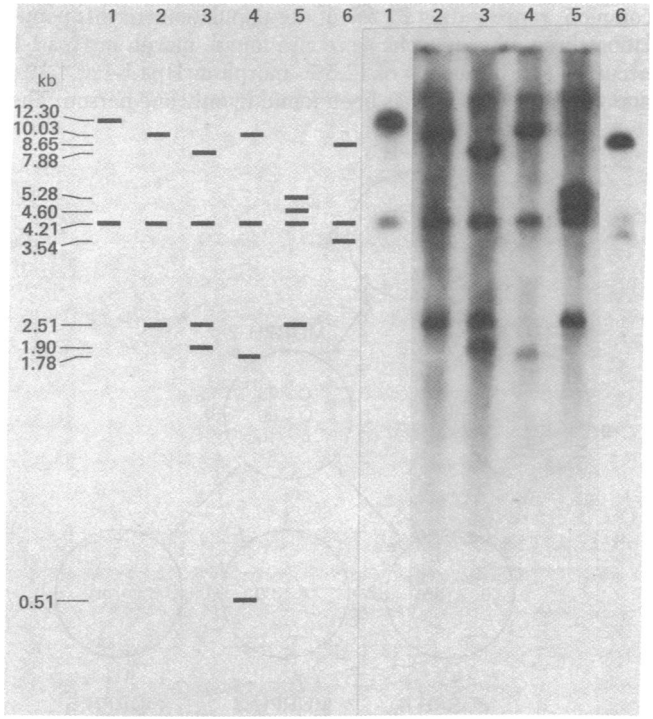

FIG. 1. Autoradiograph of *Hpa* I mtDNA fragments belonging to the different *Hpa* I morphs. Fragments were separated on a 1% gel, transferred to a nitrocellulose filter, and hybridized with [^{32}P]mtDNA (specific activity 2×10^8 cpm/μg, 3×10^6 cpm per filter). The sizes of the fragments are given on the left. Channel 1, mtHpa I-1; channel 2, mtHpa I-2; channel 3, mtHpa I-3; channel 4, mtHpa I-4; channel 5, mtHpa I-5; channel 6, mtHpa I-6. Also shown is a diagram of these different morphs. The 0.51-kb band was detected on autoradiography with longer exposure.

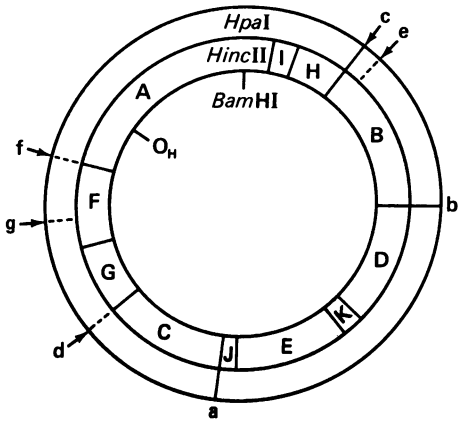

FIG. 2. Restriction endonuclease cleavage map of human mtDNA. The *Hpa* I cleavage sites (outer ring) are indicated in lower-case letters. Solid lines show the three sites already mapped, a, b, and c (11, 23, 24). The *Hinc*II fragments are designated by upper-case letters. The *Hinc*II map was adapted from Brown and Goodman (11). The *Hpa* I sites a and b were present in all mtDNA samples examined. Sites c–g were polymorphic and are indicated by arrows. The map is oriented with the single *Bam*HI site at 0, and the heavy strand origin of replication (O_H) is indicated.

recognizes the sequence G-T-T-A-A-C, whereas *Hin*cII recognizes G-T-Y-R-A-C (Y, pyrimidine nucleoside; R, purine nucleoside). Therefore all *Hpa* I sites are also *Hin*cII sites but the converse is not true.

Morph mtHpa I-1 differs from morph mtHpa I-2 by the absence of site c, as deduced from the calculation of fragment sizes as shown in Fig. 1. Site c has been localized in an open reading frame as defined by the DNA sequence (8) to which no function has been ascribed. Digestion with *Hin*cII of morph mtHpa I-1 samples indicated that the mutation was such that *Hin*cII sensitivity was also lost at this site. This agrees with results of Brown (15) and unpublished data. The remaining two sites, a and b, were found in all human mtDNAs examined. These sites are located in the anticodon loops of putative tRNAs as defined by the mtDNA sequence (8). The a site includes the asparagine tRNA anticodon sequence CAA plus the three adjacent nucleotides on the 3′ side. The b site incorporates the bases in the stem of the anticodon loop of the glycine tRNA.

Morph mtHpa I-3 differs from morph mtHpa I-2 by the presence of an additional site located in the 9.8-kb fragment delineated by sites c and a, as read counterclockwise ($\overline{ca}$). The new site occurred at a distance of 2.1 kb from one of the two ends. There was already a *Hin*cII site at exactly this distance from the a site (fragment C, Fig. 2) but not at this distance from the c site. It is therefore possible that the new site originated from a mutation in this *Hin*cII site. A *Hin*cII digestion of the two morphs, mtHpa I-2 and mtHpa I-3, shows no difference in the cleavage patterns, thus making it unlikely that a new *Hpa* I and *Hin*cII site occurred 2.1 kb from the c site. Therefore, the nucleotide substitution responsible for the transition from morph mtHpa I-2 to morph mtHpa I-3 was most likely a mutation in this *Hin*cII site to also permit recognition by *Hpa* I. This new *Hpa* I site was designated d (Fig. 2). The sequence data (8) show that the *Hin*cII site sequence is G-T-C-A-A-C. Therefore the mutation must have been a transition, C to T, in the third nucleotide to yield the *Hpa* I site G-T-T-A-A-C. This region codes for an unknown protein with the first three nucleotides of the *Hpa* I site coding for valine (GTC). The observed sequence change would create codon GTT, which also codes for valine (5). Hence, the observed mutation would not affect the polypeptide sequence and thus would presumably be selectively neutral.

Morph mtHpa I-4 also has four fragments, two of which ($\overline{ab}$ and $\overline{ca}$) are the same as in morph mtHpa I-2. Therefore, the two new fragments must have been generated by a new restriction site in fragment $\overline{bc}$. This site was mapped by a double digestion with *Hin*dIII and *Hpa* I (data not shown) and was located at position e shown in Fig. 2. The site e has been localized in an open reading frame as defined by the sequence (8) and its effect on the hypothetical protein is unknown.

Morph mtHpa I-5 has two fragments, $\overline{ab}$ and $\overline{bc}$, common to morph mtHpa I-2, while the fragment $\overline{ca}$ has been split into two new fragments, $\overline{cf}$ and $\overline{fa}$. To localize this new site the *Hin*cII map was again useful. *Hin*cII digestion showed no difference from the typical *Hin*cII pattern. Therefore, on the basis of the fragment sizes, the *Hin*cII site in the region of the 12S RNA, defining *Hin*cII fragment F, and on the side of the origin of replication of the heavy strand (O_H), most likely mutated to create a new *Hpa* I site, designated f.

Morph mtHpa I-6 has three fragments, one of which, $\overline{ab}$, corresponds to that observed in all other morphs. The new site could be located on either side of O_H. By *Hin*cII digestion we observed a new *Hin*cII site in the fragment (F), and on the basis of fragment size we mapped this as the *Hpa* I g site (Fig. 2), which occurred in the 16S rRNA.

Frequency of the Morphs in Different Populations. The six morphs were found to occur in widely different frequencies in the populations tested (see Table 2). The average ethnic group sample was 45 individuals, and the total number of individuals examined was 235. The most common morph and the one present in all the populations tested was mtHpa I-2. In Caucasians only one other morph, mtHpa I-5, was found, and then only in one person. This rare Caucasian morph was not found in any other population tested.

In the Oriental populations, morph mtHpa I-2 was the most common, representing 81.5% of the population. In this population three other morphs were also found: morph mtHpa I-1 occurred at a frequency of 12.5%, morph mtHpa I-4 at 4.1%, and morph mtHpa I-6 has been found in only one person. The

Table 2. Frequencies of mtHpa I morphs for different populations

Population	Sample size	Frequency, % Morph I-1	Morph I-2	Morph I-3	Morph I-4	Morph I-5	Morph I-6
Bushman	41	0	7.3	92.7	0	0	0
Pygmy	44	0	4.5	95.5	0	0	0
Bantu	48	4.2	25.0	70.8	0	0	0
African*	133	1.5	12.8	85.7	0	0	0
Oriental	48	12.5	81.3	0	4.2	0	2.1
Caucasian	54	0	98.1	0	0	1.9	0
Total	235	3.4	46.4	48.5	0.9	0.4	0.4

Significant level of differences has been tested by using the χ^2 test among any pairs of three African ethnic groups and among any pairs of Africans, Orientals, and Caucasians. At the level of 95% the following pairs are significantly different: Pygmies and Bantus, Africans and Orientals, Africans and Caucasians, Orientals and Caucasians, and Bantus and the pooled frequency of Pygmies and Bushmen.

* African frequencies are the average of the three ethnic groups (Bushman, Pygmy, and Bantu).

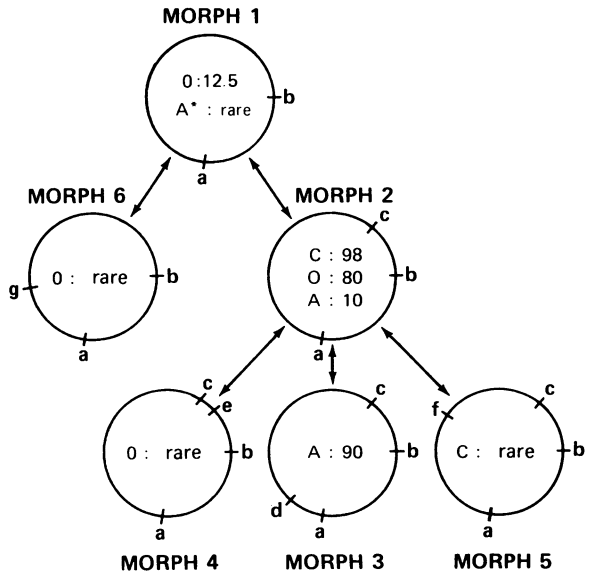

FIG. 3. Phylogeny of the mtHpa I morphs found in this study. The lower-case letters on the rings indicate the restriction sites mapped in Fig. 2. Within each circle the frequencies of that morph for the different populations are indicated. The frequencies reported for the African samples are the means of the three ethnic groups studied. The three rows of circles have been grouped according to those having 2, 3, and 4 fragments, respectively. C, Caucasian; O, Oriental; A, African; and A*, Bantu (morph mtHpa I-1 has been found in only two Bantu samples of the African group).

last two rare Oriental morphs were not observed in the other populations tested. The frequency of mtHpa I-2 was significantly different in this population compared to the frequency in Caucasians (see Table 2).

In the African populations morph mtHpa I-2 was rare, accounting for only 7% of Bushmen and 4.5% of Pygmy mtDNAs. In contrast, a different morph, mtHpa I-3, was found only in African populations and was present in greater than 90% of mtDNAs tested in these groups. The difference in frequency of morph mtHpa I-2 between these two African populations and Caucasians and Orientals was the most dramatic difference found. As such it provides an excellent marker for differentiation of these populations. In this regard, the frequency of mtHpa I-2 is significantly higher in Bantus than in the two other African groups, a fact in agreement with the pattern observed for other classical markers (25). Also, two Bantu samples (4%) were found to have morph mtHpa I-1, the origin of which is unclear.

Fig. 3 shows that each of the morphs can be related to each other by single *Hpa* I site changes.

DISCUSSION

The analysis of *Hpa* I cleavage patterns in mtDNA of 235 individuals from five ethnic groups has revealed remarkable variation between individuals. Six different morphs were found, which could be shown to be derived one from the other by single changes believed to be point mutations. The diagram of Fig. 3 shows their phylogenetic relationships on this assumption. One of these does not involve amino acid substitution and is therefore presumably neutral. Two others affect the ribosomal RNA genes only. The most common morph in all populations except Africans was composed of three fragments and could generate four of the other patterns by single base change. Three of these have four fragments and one (morph mtHpa I-1) has only two. The sixth morph has three fragments and could have originated only by a single mutation from morph mtHpa I-1.

These data alone would not be sufficient to reconstruct in full the temporal sequence of events. However, recent information on other primates (26) has revealed that sites a and b are found in all cases, and morph mtHpa I-1 containing these two sites is identical to that of orangutans. The high degree of conservation of these two sites may be related to their location in the tRNA structural genes. None of the other *Hpa* I sites are convincingly present in the other primate groups, which all have their own unique sites. These data permit the construction of a tree that relates the different *Hpa* I morphs to each other by a minimum number of mutations (Fig. 4). This tree suggests that morph mtHpa I-1 (with two sites) is the original human morph, though other hypotheses could be proposed. Morph mtHpa I-2, the most common one, could be ancestral and give rise to the other morphs. Alternatively, morph mtHpa I-3 (or others) could be ancestral. However, these other models require a greater number of mutations to relate the human patterns to those observed in the other primates. Until more apes are tested, one cannot exclude the possible existence of polymorphisms in these species that might complicate this interpretation.

If morph mtHpa I-1, which is identical to the basic pattern found in all apes, is ancestral for hominoids, it is of considerable interest that this morph was found only in Orientals and Bantus. The most likely source of the original mtDNA pattern for the formation of the human ethnic groups seems to be Orientals. This proposal is supported by three observations. First, morph mtHpa I-1 was found in a higher frequency in Orientals (12.5%) than in Bantus (4%). Second, morph mtHpa I-6 found only in Orientals can be related to the others only through morph mtHpa I-1. Finally, if Bantus were central to the radiations of the ethnic groups, then it is most surprising that morph mtHpa I-1 was not found in either Pygmies or Bushman. Rather, it seems more likely that the presence of morph mtHpa I-1 in Bantus is due to a more recent admixture.

The suggestion for these mtDNA data that the human ethnic groups diverged from an Asian origin is consistent with comparable data on nuclear gene frequencies. These results suggest that formation of human ethnic groups took place in the last part of the Pleistocene, starting in Asia and radiating towards the other continents, probably within the last 50,000–100,000 years (27). An estimate of the divergence of human ethnic groups based on mtDNA variation has been made in the range of 10,000–50,000 years (M. Nei, personal communication), correcting a previous estimate (12) and in agreement with an earlier estimate of 25,000–100,000, based on classical genetic markers (28). The hypothesis of an Asian origin of man has been independently advanced on the basis of the presence of type C virus in Asian apes and man (29).

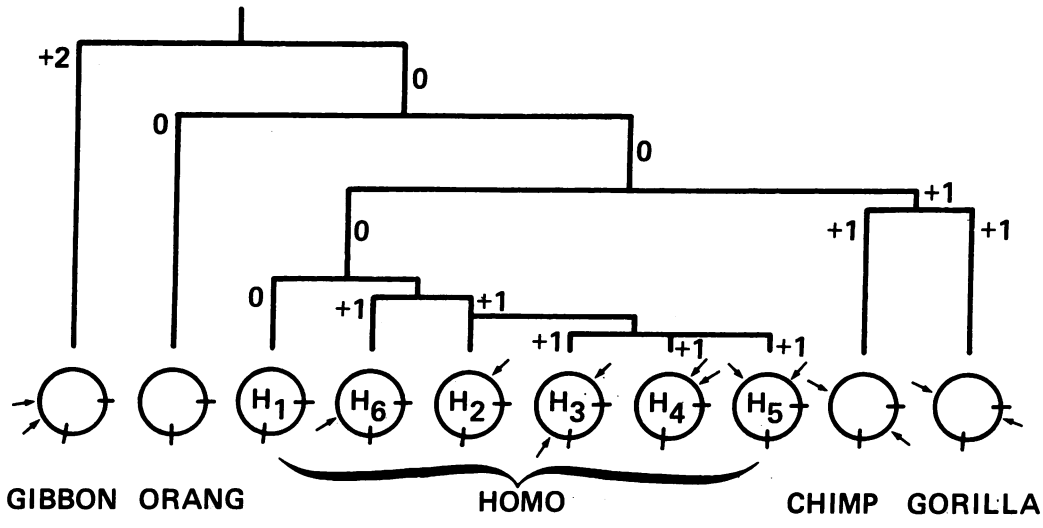

FIG. 4. Minimum mutation tree including human and primate data for *Hpa* I. The primate data were obtained from Ferris *et al.* (26). The numbers represent the number of base changes necessary to derive the next morph. Ticks on the circles show the positions of the invariant sites, and arrows show those of the variant sites.

If morph mtHpa I-1 is truly the ancestral type, this morph must have been substituted and almost completely replaced by morph mtHpa I-2 before the radiaton from Asia. If this interpretation is correct, morph mtHpa I-2 itself must have undergone a further mutation to morph mtHpa I-3, presumably in Africa, and the latter morph must have subsequently increased substantially in frequency in these populations. Because the mutation of morph mtHpa I-2 to mtHpa I-3 seems to be neutral, this evolutionary change probably occurred by random genetic drift unless it was coupled with some other mutation that is itself selected for. Other interpretations are possible, but demand recurrences of mutations, which are in general less likely. The evolutionary picture emerging from the results with this enzyme is clear. We look forward to tests with other enzymes and other ethnic samples to see if it will be confirmed.

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